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A Spectrophotometric Assay for Total Garlic Thiosulfinates Content. Kinetic Aspects of Reaction with Chromogenic Thiols

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Garlic (*Allium sativum*) is a plant known for its antimicrobial, antithrombotic and antiatherosclerotic properties. Those features are attributed to thiosulfinates, compounds formed in an enzymatic reaction occurring during garlic cloves crashing.

A spectrophotometric method for the determination of thiosulfinate concentration in garlic extracts and supplements was proposed. It is based on reactions between thiosulfinates and selected chromogenic thiol compounds: 2-mercaptopyridine (2-MP), 4-mercaptopyridine (4-MP), 1-oxide-2-mercaptopyridine (MPO), 2-mercaptopyrimidine (MPM) and 2-nitro-5-thiobenzoic acid (NTB). Then kinetics of the reactions was studied. Rate constants and orders were determined. All reactions are first order with respect to the thiol and first order with respect to TS so the reactions of all tested thiols with garlic TS run in accordance with second order kinetics. Reaction rates vary among the thiol compounds: the constant rates differ from 6.7 $(mol/L)^{-1}.min^{-1}$ for 2-MP to 1129 $(mol/L)^{-1}.min^{-1}$ for NTB.

Knowledge of the kinetic equation for the reactions and measurements of the initial rates allowed determining thiosulfinate contents in garlic extracts and supplements.

INTRODUCTION

Garlic (*Allium sativum*) is a plant known for its medicinal properties. Also, its cloves are used for consumption in various regions of the world, either raw, cooked or fried. Garlic is applied to spice dishes due to its specific pungent flavour and used to preserve meat and vegetables because of its antimicrobial properties. Intact garlic bulbs contain organosulfur compounds, particularly γ-glutamylcysteines and cysteine sulfoxides. Within the garlic bulb, during storage, a part of the γ-glutamylcysteines convert into the cysteine sulfoxides which are precursors of thiosulfinates (TS). Thiosulfinates are formed during an enzymatic reaction catalysed by alliinase (*alliin lyase*, EC 4.4.1.4):

$$\begin{aligned} &R\text{-}S(O)\text{-}CH_2\text{-}CH(NH)_2\text{-}COOH + H_2O \rightarrow \\ &\rightarrow R\text{-}SOH + CH_3\text{-}C(O)\text{-}COOH + NH_3 \\ &R\text{-}SOH + R_1\text{-}SOH \rightarrow R\text{-}S(O)\text{-}S\text{-}R_1 + H_2O \end{aligned} \quad (1)$$

At first pyruvic acid, ammonia and alk(en)yl sulfenic acid are formed. Afterwards rapid condensation of sulfenic acids occurs and thiosulfinates $R\text{-}S(O)\text{-}S\text{-}R_1$ are formed, where R and R_1 represent methyl, 1-propenyl or 2-propenyl group.

The reaction occurs during garlic cloves crushing. There are eight known dialk(en)yl thiosulfinates in crushed garlic: allyl-2-propenethiosulfinate (allicin) is the most abundant (50–90%mol). The others are: allylmethaneTS (3–20%mol), *trans*-1-propenyl-2-propeneTS (5–18%mol), methyl-2-propeneTS (1.5–8%mol), allyl*trans*-1-propeneTS (1.5–2%mol), methylmethane-TS (1–2%mol), *trans*-1-propenylmethaneTS (1–2%mol), methyl-*trans*-1-propeneTS (0.5%mol) [Block, 1992; Kyung & Lee, 2001; Lawson & Gardner, 2005].

The characteristic odour of garlic and many of its health beneficial properties: antimicrobial, antithrombotic and antiatherosclerotic, are attributed to thiosulfinates strong reactivity to free -SH groups and its antioxidant properties [Qi & Wang, 2003; Juszkiewicz *et al*., 2004; Hiyasat *et al*., 2009]. Although allicin, the enzyme alliinase and the reaction of TS formation have been known and examined since 1944 [Cavallito & Bailey, 1944], a simple and sensitive method of quantitative TS determination is still being searched. GC and HPLC methods are used for quantitative determination of TS [Rosen *et al*., 2001; Arnault *et al*., 2003] but for GC analysis allicin and other thiosulfinates must be converted to more stable compounds, not so sensitive to high temperature as TS. The problem does not occur when HPLC is used because analyses are taken at room temperature. However, low resolution and the necessity of using external standards limit the applicability of GC and HPLC methods. Enzymatic biosensors method may also be used for TS determination [Krest & Keusgen, 2002; Keusgen *et al*., 2003]. This method allows not only to determine TS concentration but also to monitor the course of the enzymatic reaction. The analysis is quick

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and precise and does not require sophisticated equipment, but it is difficult to obtain the enzyme.

For measurement of TS concentration spectrophotometric methods were also tested. Two kinds of compounds may be applied: directly or indirectly allowing to determine TS content in a UV-Vis range. The first group includes thiols (compounds with sulphydryl groups -SH), *e.g.* NTB [Miron *et al*., 1998] and 4-MP [Miron *et al*., 2002]. These compounds react with a disulfide bond of thiosulfinates. The course of the reaction is observed by monitoring thiols concentration. For indirect spectrophotometric determination disulfide acid compound 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was used by Han *et al*. [1995]. This assay is based on the reaction of TS with excess of cysteine. The residue of cysteine reacts with the disulfide acid. As a result of the reaction, a coloured compound absorbing in the UV-Vis range is formed. Another way of the quantitative TS determination is to monitor contents of ammonia and pyruvic acid, the other products formed during the enzymatic reaction (Equation 1). However, both of them are present in garlic cloves before crushing [Yoo & Pike, 2001], hence it is necessary to take into consideration the background of ammonia and pyruvic acid concentrations in the plant.

In view of the above, we propose a new spectrophotometric method for the determination of thiosulfinates content, based on their reaction with chromogenic thiols. It requires knowledge of the reaction kinetics and determination of rate constants. The method allows determining the total concentration of thiosulfinates in garlic and its supplements. It is important to know whether the supplements contain any thiosulfinates and what is their content because the compounds beneficial for health might be destroyed during production.

MATERIALS AND METHODS

Chemicals

2-mercaptopyridine (2-MP) and 4-mercaptopyridine (4-MP) were purchased from Aldrich, whereas 1-oxide-2-mercaptopyridine (MPO) and 2-mercaptopyrimidine (MPM) were purchased from Fluka Chemika. 2-nitro-5-thiobenzoic acid (NTB) was prepared according to Degani & Patchornik [1971], whereas L-cysteine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 2,2'-dithiodipyridine (DTDP) were purchased from Sigma. The chemicals were of analytical grade. Structural formulas of thiol compounds used in the study are presented in Figure 1.

Preparation of plant material

The garlic used was from China. Its cloves were ground with a mortar and mixed with water: 10 mL per 1 g of garlic [Lawson & Gardner, 2005]. Next, the suspension was shaken for 30 min and filtered through gauze. Then undissolved material was removed by centrifugation at 300×*g* for 4 min.

Preparation of garlic supplements

Garlic supplements: Biogarlic (Pharma Nord, Danmark), Garlic Extract (Unipharm, USA), and Pulverised Garlic (Solgar, USA) – all available in Polish pharmacies, were tested. Aqueous extracts of garlic supplements were prepared according to Lawson & Wang procedure [2001]: weighted tablets were ground with a mortar and mixed with water: 50 mL per 1 g of tablet. Then the suspensions were shaken for 30 min, filtered through gauze and centrifuged at 300×*g* for 4 min.

UV-Vis spectra

Absorption spectra of tested thiols and their changes during the reaction with thiosulfinates were recorded using Marcel Media (Marcel, Zielonka, Poland) UV-Vis spectrophotometer in a 330-550 nm range for about 100 μmol/L thiols solutions in 50 mmol/L phosphate buffer, pH=7.0 and 1 mmol/L EDTA.

Quantitative determination of TS concentration

The aqueous garlic extracts were initially standardised for their thiosulfinate concentration using the spectrophotometric method of Han *et al*. [1995]. This method is based on the fact that one molecule of thiosulfinate reacts with two molecules of cysteine to form two molecules of *S*-alkenyl or *S*-alkylmercaptocysteine. The drop of cysteine content is measured spectrophotometrically at 412 nm in the form of 2-nitro-5-thiobenzoic acid obtained in a reaction with DTNB. Residual concentration of cysteine was determined also by the reaction with DTDP, followed by the measurement of 2-mercaptopyridine concentration at 343 nm.

Kinetics examination of the reactions between chromogenic thiols and thiosulfinates

Absorbance measurements were carried out in a 1 cm cuvette using the Marcel Media UV-Vis spectrophotometer (330–800 nm range). In the kinetic measurements, 1 mL of the TS solution was added and mixed with 1 mL of the thiol solution and then absorbance changes were recorded every 10 or every 2 minutes. In calculations, stirring time was taken into account. Reactions were carried out in a phosphate buffer, pH=7.0 and 1 mmol/L solution of EDTA at room temperature. Absorbance was measured at λ_{max} for each thiol that results in minimisation of absorbance measurement error. For MPO and MPM molar absorbance coefficients are low, therefore there were used more concentrated solutions: [MPO] = 250 μmol/L and [MPM] = 510 μmol/L. For the other thiol solutions the concentration was about 100 μmol/L. In all cases, concentrations refer to solutions after mixing.

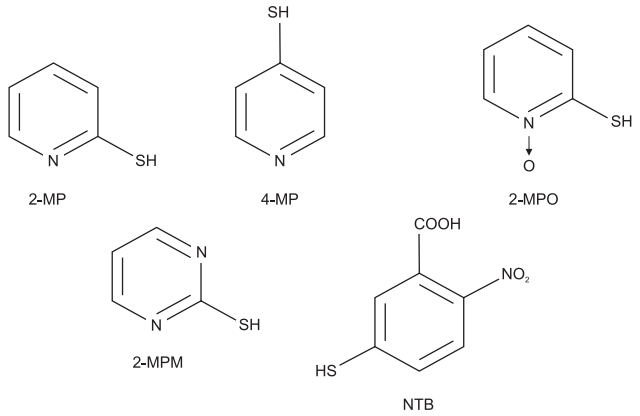

FIGURE 1. Chromogenic thiols used in the study.

RESULTS AND DISCUSSION

Total thiosulfinates concentration in garlic extracts

Thiosulfinates content in garlic cloves depends on conditions of plant cultivation: insolation, temperature, humidity of soil, and also depends on storage conditions of garlic bulbs [Ichikawa *et al*., 2006]. For this reason, the concentration of thiosulfinates in the tested garlic extracts was examined using the Han *et al.* [1995] method before each kinetic measurement. Unreacted cysteine was determined in a reaction with DTNB and with the other disulfide compound: 2,2'-dithiodipyridine (DTDP) which has not been applied before. Measurements were repeated 10-fold. TS concentration was calculated as an arithmetical mean of both methods.

Thiosulfinates content of the examined garlic extracts was in a concentration interval from 3.1 to 3.6 mmol/L with precision of concentration determination equal to 3%. The modified cysteine method, using DTDP in its second step, gave results which were comparable to the classic Han method results. It means the results of both methods are equal within the range of one standard deviation. An advantage of the modified method is in greater stability of the reaction mixture that allows measurements in a wider pH range, *i.e.* from 2.0 to 8.0, while the use of DTNB allows measurements only in a 7.5 to 8.5 pH range [Egwim & Gruber, 2001; Riener *et al*., 2002]. The method using 2,2'-DTDP may be useful in analyses under simulated gastrointestinal conditions.

UV-Vis absorption spectra of chromogenic thiols

UV-Vis absorption spectra were recorded for solutions of the chromogenic thiols. The molar absorbance coefficients determined from the spectra and known from literature as well as λ_{max} for each thiol were collected in Table 1. Absorption spectra of two tested thiols, NTB and 2-MP, recorded during their reaction with garlic thiosulfinates are shown in Figure 2. Only for NTB a band derived from the reaction product was observed in the examined range.

Reaction of thiosulfinates from garlic extracts with the chromogenic thiols

On the basis of Miron *et al.* [1998, 2002] studies it is known that thiols: 4-MP and NTB react with allicine (stoichiometric ratio 2:1) by -SH group forming a disulfide bond. In the first case 4-*S*-allylmercaptothiopyridine, wheras in the second case *S*-allylmercapto-5-thio-2-nitrobenzoic acid is formed. Taking into account the previous studies, the following reaction may be proposed for the studied chromogenic thiols and garlic TS:

$$2\text{P-SH} + \text{R}_1\text{-S(O)-S-R}_2 \rightarrow \\ \rightarrow \text{P-S-S-R}_1 + \text{P-S-S-R}_2 + \text{H}_2\text{O} \qquad (2)$$

where: P-SH – a thiol reagent; R_1, R_2 – alk(en)yl groups: methyl, 1-propenyl or 2-propenyl; and P-S-S-R_1, P-S-S-R_2 – alk(en)yl disulfides.

TABLE 1. Spectral characteristics of the chromogenic thiols and rate constants for their reactions with garlic thiosulfinates.

Thiol	λ_{max}/ nm	ε / ((mol/L)$^{-1}$. cm^{-1})	Rate constant *k* / ((mol/L)$^{-1}$. min^{-1})
2-mercaptopyridine	343	6680	6.7±0.4
4-mercaptopyridine	324	19800 *	69±3
1-oxide-2-mercaptopyridine	330	3860	73.2±0.3
2-mercaptopyrimidine	336	1796	104±1
2-nitro-5-thiobenzoic acid	412	14150 **	1129±13

* [Miron *et al.*, 1998]; ** [Lawson & Gardner, 2005]

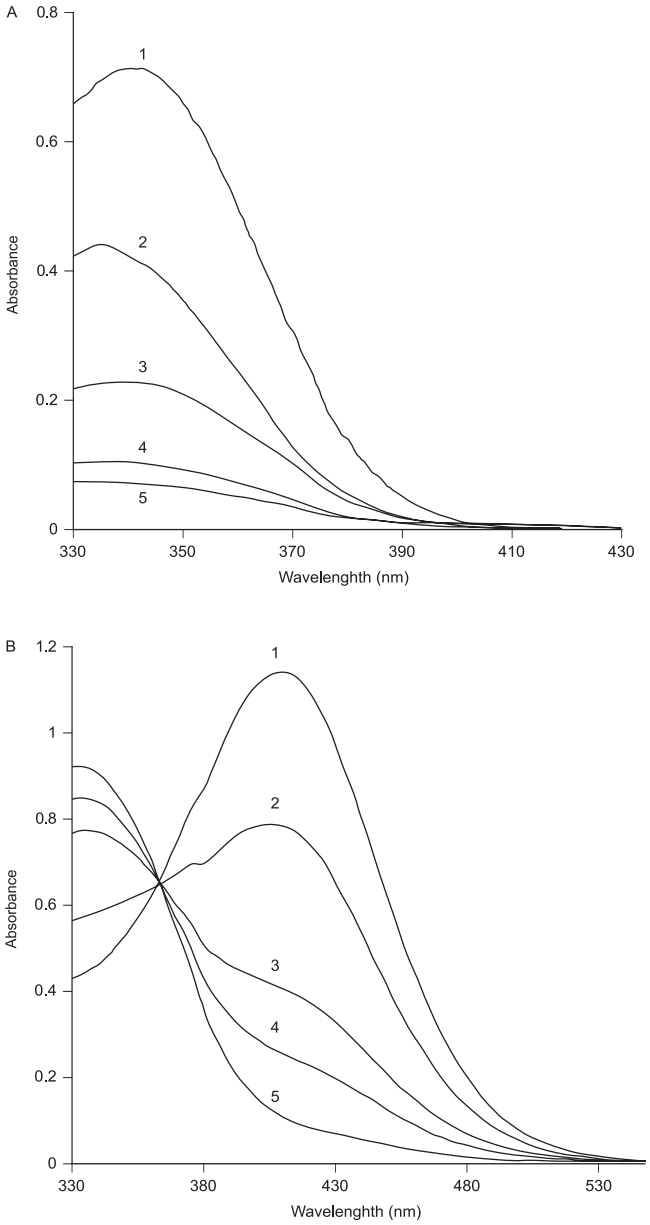

FIGURE 2. UV-Vis absorption spectra of 2-mercaptopyridine (A) and 2-nitro-5-thiobenzoic acid (B) during the reaction with thiosulfinates recorded at different time:
A 1 – before the reaction, 2 – after 40 minutes of the reaction, 3 – 120 min, 4 – 230 min, 5 – 420 min
B 1 – before the reaction, 2 – after 1 minute of the reaction, 3 – 6 min, 4 – 10 min, 5 – 23 min
The initial concentrations of the solutions were:
A – [TS] = 750 μmol/L , [2-MP] = 105 μmol/L
B – [TS] = 110 μmol/L , [NTB] = 80 μmol/L

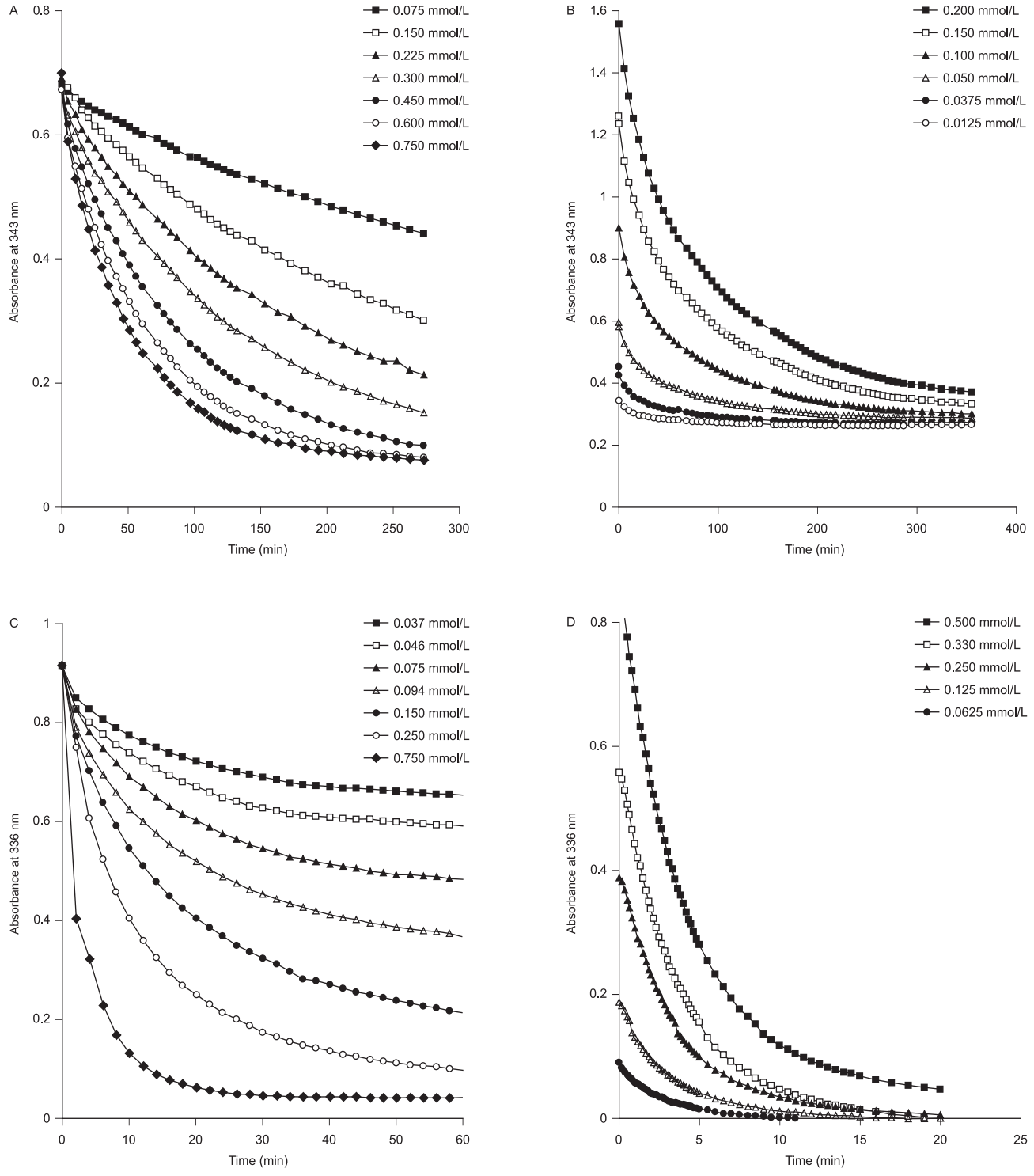

FIGURE 3. Kinetics of the reactions of garlic thiosulfinates with 2-mercaptopyridine (A,B) and 2-mercaptopyrimidine(C,D) .
A – [2-MP] = 105 μmol/L , TS concentrations given in the figure
B – [TS] = 750 μmol/L, 2-MP concentrations given in the figure
C – [MPM] = 510 μmol/L, TS concentrations given in the figure
D – [TS] = 750 μmol/L, MPM concentrations given in the figure

For all tested chromogenic thiols courses of their reactions with thiosulfinates from garlic extracts were recorded. Figures 3 and 4 depict the courses of selected thiol compounds. The reaction of 2-MP with TS was the slowest and therefore it was recorded for more than 4 h, the reaction of NTB with TS was the fastest and it was registered for 30 min. Figure 3A shows the course of reaction between thiosulfinates of garlic extract solutions of different concentrations and 2-MP solution of constant concentration. Figure 3B shows the situation when TS solution concentration was constant and TS reacted with 2-MP solutions having different concentrations. Similar kinetic curves were recorded for MPM and NTB (Figures 3CD, 4A). TS with NTB reaction was also monitored by absorbance measurement of the product at λ=330 nm. The absorption band of the product can be observed in the spectrum in Figure 2B. The shape of substrate

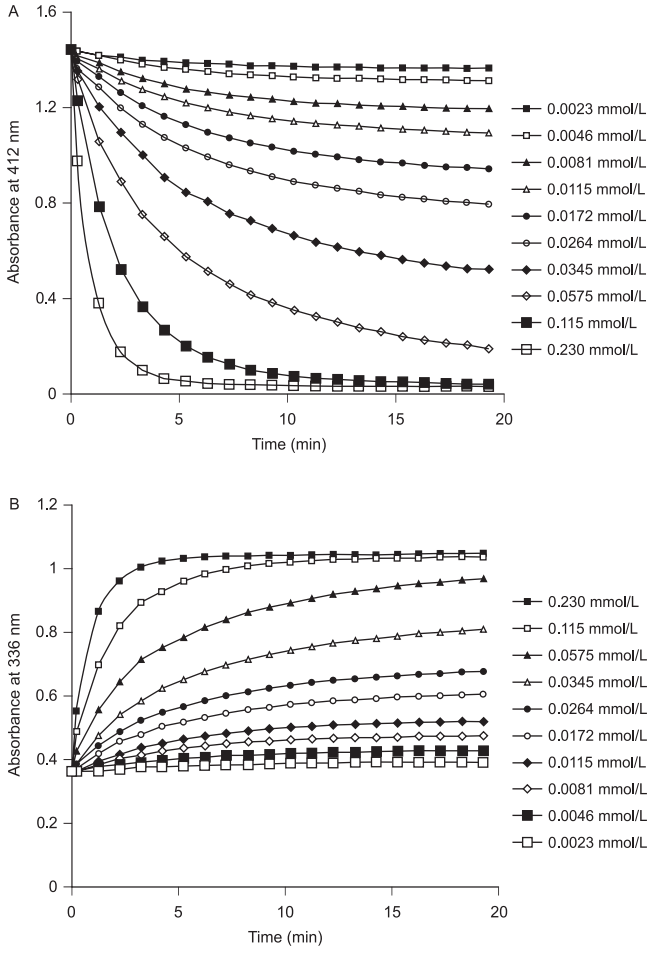

FIGURE 4. Kinetics of the reaction between 102 μmol/L solution of 2-nitro-5-thiobenzoic acid and garlic thiosulfinates. Concentrations of TS are given in the figures. The reaction course monitored at 412 nm (A) and 336 nm (B).

and product kinetic curves (Figure 4AB) confirms the supposed reaction stoichiometry.

In an independent experiment initial rates υ_0 of the above-mentioned reactions were determined. Concentration changes of the tested thiols were measured every 10 s for 10 minutes. In the period of time when dependences of absorbance upon time were linear, the initial rates are equal slopes of lines $A(t)$.

Reaction order with respect to thiols and to thiosulfinates

Reactions' orders were determined using the isolation method: initial rate υ_0 of TS – thiol reactions were obtained applying an excess of thiol in one experiment and then applying TS excess in the second one. From the linear dependence of log υ_0 upon log [TS], reaction order with respect to TS was calculated as a slope. From the linear dependence of log υ_0 upon log [P-SH], reaction order with respect to the thiol was calculated in the same way. An example of orders of determination for TS – NTB reaction is shown in Figure 5. The orders of the reaction between all the tested thiols and garlic TS were determined in a corresponding way. All reactions are first order with respect to the thiol and first order with respect to TS so the total order of the reaction is two.

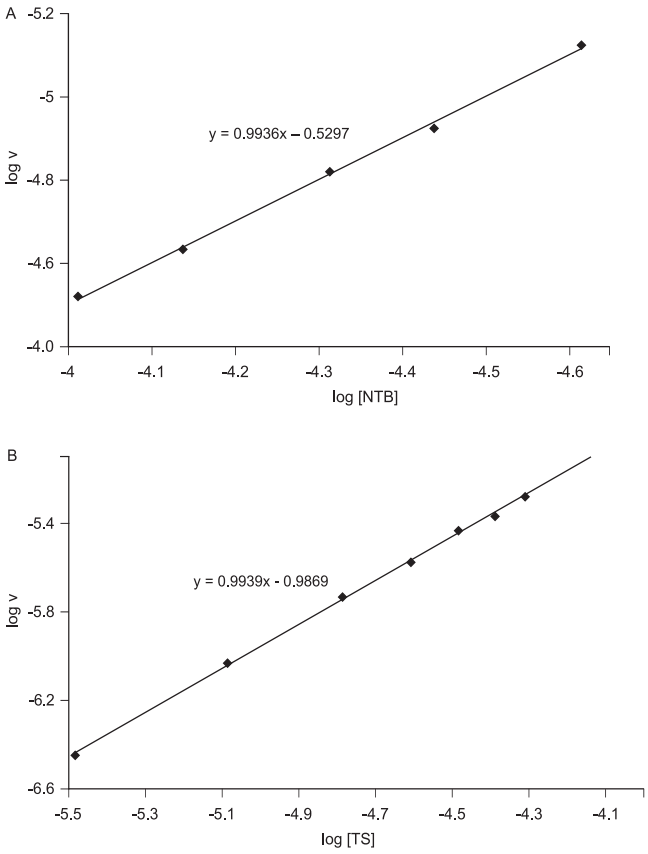

FIGURE 5. Determination of the order of reaction between NTB and garlic thiosulfinates using the initial rate method.
A – order with respect to NTB
B – order with respect to TS

Rate constants

Rate constant of the reaction was calculated using an expression for second order reaction:

$$k = \upsilon_0 / ([\text{P-SH}]^1 \cdot [\text{TS}]^1) \quad (1F)$$

For the initial linear fragment of each kinetic curve the reaction initial rate υ_i was determined as the slope of $A(t)$ line. Table 2 shows an example of determination of rate constant for MPM – TS reaction. Rate constants for all tested chromogenic thiols were collected in Table 1. The constant rate of the reaction was calculated as an arithmetical mean of rate constants obtained for different TS or thiol concentrations. Uncertainties of rate constants were calculated as standard deviations of the means.

The chromogenic thiols method

Analysis of the kinetic curves allowed to determine the initial rates and rate constants of the reaction. The partial orders were also estimated with respect to the thiols and thiosulfinates, indicating that the kinetic equation for all tested reactions is as follows:

$$\upsilon = k \cdot [\text{P-SH}]^1 \cdot [\text{TS}]^1 \quad (2F)$$

Reactions of all tested thiols with garlic TS ran in accordance with second order kinetics, but their rates were differ-

TABLE 2. Initial rates υ_{0i} and rate constants for the reaction between garlic thiosulfinates and 2-mercaptopyrimidine.

$[TS]_0$ / µmol/L	$[MPM]_0$ / µmol/L	Initial rate $(\Delta A_i/t_p)$ / (min^{-1})	Initial rate υ_{0i} / 10^{-6}(mol/L). min^{-1}	Constant rate k / $((mol/L)^{-1}\cdot min^{-1})$
28.8	500	0.00543	1.15	105
33.4	500	0.00654	1.82	109
38.4	500	0.00695	1.93	101
57.7	500	0.01137	3.17	110
72.2	500	0.01457	4.06	112
87.5	500	0.01627	4.53	104
116	500	0.01933	5.38	93
144	500	0.02783	7.75	107
175	500	0.03383	9.42	108
292	500	0.05127	14.3	98
578	500	0.09728	27.1	94
875	500	0.15355	2.7	98
350	62.5	0.00866	2.41	110
350	125	0.01653	4.60	105
350	250	0.03214	8.95	102
350	330	0.04363	12.1	104
350	500	0.06567	18.3	104

ent (Table 1). From the reaction kinetics, the rate constant and the kinetic equations, TS concentration can be obtained by measuring absorbance decrease, ΔA_p in the first phase of the reaction:

$$\Delta A_p = A_0 - A(t_p) \quad (3F)$$

where t_p is the reaction time so short that the $A(t)$ curve can be approximated to a line. Then:

$$[TS]_0 = \upsilon_0 / (k \cdot [P\text{-}SH]_0), \quad (4F)$$

$$\text{where } \upsilon_0 = \Delta A_p / (2 \cdot l \cdot \varepsilon_{P\text{-}SH} \cdot t_p) \quad (5F)$$

The proposed method of TS concentration determination is based on the measurement of chromogenic thiols absorbance decrease. Because of that, the determination is the more precise the greater is the measured decrease in ΔA_p. If the determination is made using the initial rates method they cannot be too large because it is difficult to measure them. But, on the other hand, they cannot be too small because a big error is made or it takes too long time to measure υ_0. It is obvious that rates of tested reactions depend not only on the rate constants but also on the TS concentration. So, when a low concentration is determined it is better to use a thiol reagent which rapidly reacts with thiosulfinates, and in the case of high expected TS concentration – a slow-reacting thiol.

Using the proposed thiol method the TS content was determined for several garlic supplements purchased from Polish pharmacies. Uncertainties of TS concentration determination for 2-MP and 4-MP were about 5%. They mainly resulted from uncertainties of rate constants determination.

TABLE 3. The comparison of thiosulfinates content in tested garlic supplements.

Content of TS	Garlic Extract (Pharma Nord)	Biogarlic (Unipharm)	Pulverized garlic (Solgar)
TS content (µmol per 1 g of supplement)	7.2±0.3	17.9±0.9	38.0±1.3
TS content (mg allicin per tablet)	0.835±0.034	2.09±0.08	2.22±0.08
Producer-indicated TS content (mg allicin per tablet)	1.0	2.1	1.5

For the other thiols used, the uncertainty of TS concentration determination is about 3% and it is caused of initial rates uncertainty. To minimise them thiol and thiosulfinates solutions should be diluted in such a way that the decrease of absorption ΔA_p equals at least 0.3.

A comparison of the results and TS content indicated by the producers for the tested garlic supplements is shown in Table 3. Only for a few supplements the comparison was possible because others, available in Polish pharmacies, were not standardised in terms of the content of allicin nor of other thiosulfinates. Sometimes only the mass of garlic used for one tablet preparation is indicated. However, it is not reliable because of the mentioned above dependence of TS content in garlic on cultivation and storage conditions. The proposed chromogenic thiols method may be useful for standardisation, because it is important to know the actual contents of active compounds in applied garlic supplements.

CONCLUSION

On the basis of the research we postulate that almost every chromogenic thiol is applicable for quantitative TS determination in fresh garlic extracts and supplements. It is only necessary to record the thiol UV-Vis spectrum. Knowing its λ_{max} it is possible to monitor the course of its reaction with TS and to determine the rate constant of the reaction. The proposed method enables determining the total concentration of thiosulfinates, not only of allicin. This is more accurate in the case of fresh garlic and its supplements because allicin content is flexible and mutual proportions of all TS are variable and depend on cultivation and storage conditions [Ichikawa *et al*., 2006]. For this reason, it is difficult to conclude on TS content only on the basis of allicin content. Moreover, it is known that not only allicin, but also all thiosulfinates have medicinal properties. Because of this, the proposed method is best suited for the determination of medicinal properties of given garlic supplement.

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